THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 21, Issue of July 25, pp. 10035-10038, 1987
Printed in U.S.A.

Sequence from Picomole Quantities of Proteins Electroblotted onto Polyvinylidene Difluoride Membranes*

(Received for publication, January 13, 1987)

Paul Matsudaira

From the Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

Small amounts (7–250 pmol) of myoglobin, β-lactoglobulin, and other proteins and peptides can be spotted or electroblotted onto polyvinylidene difluoride (PVDF) membranes, stained with Coomassie Blue, and sequenced directly. The membranes are not chemically activated or pretreated with Polybrene before usage. The average repetitive yields and initial coupling of proteins spotted or blotted into PVDF membranes ranged between 84–98% and 30–108% respectively, and were comparable with the yields measured for proteins spotted onto Polybrene-coated glass fiber discs. The results suggest that PVDF membranes are superior supports for sequence analysis of picomole quantities of proteins purified by gel electrophoresis.

Developments in protein sequencing (reviewed in Ref. 1) such as the incorporation of gas-phase methods (2), absorption of proteins onto Polybrene-coated supports (2, 3) or covalent attachment of proteins to derivatized glass (4), and on-line microbore HPLC[1] identification (1) of the PTH-derivatives have combined to permit routine sequence analysis from picomole quantities of protein. Although proteins can be isolated by electroelution from polyacrylamide gels or by HPLC, the yields are often extremely low, and the techniques involved are laborious. One improvement has been to electroblot proteins purified by gel electrophoresis directly onto glass fiber sheets (5, 6). While this approach simplifies sample handling, it is limited in its usefulness because the proteins are difficult to detect, the glass fiber filters must be derivatized, and the reagents specially purified. In addition, a recent study (7) has found that both the repetitive yield and initial yield for coupling from proteins electroblotted onto glass fiber sheets decrease with decreasing amounts of protein. As a result, small quantities of protein (<100 pmol) have still proved difficult to sequence.

Polyvinylidene difluoride (PVDF) membranes are mechanically strong solid phase supports that bind proteins hydrophobically and have been used in immunoblotting applications as a substitute for nitrocellulose filters (8). Because these membranes are inert to most solvents (acetonitrile, trifluoroacetic acid, ethyl acetate, trimethylamine, and hexane), they can be used in automated gas-phase sequenators. Here, I report that 7–250 pmol of protein can be spotted or electroblotted onto PVDF membranes, detected by staining with Coomassie Blue, and then sequenced directly. The initial coupling and repetitive yields are much higher than those reported for proteins electroblotted onto glass fiber filters. Since there is no need for preconditioning steps, elaborate derivatization of the membranes, or special purification of reagents, PVDF membranes are an ideal solid-phase support for sequence analysis, especially when used with electroblotting methods.

EXPERIMENTAL PROCEDURES

Materials and Reagents—PVDF membranes (Immobilon Transfer), 0.45-μm pore size, were obtained from Millipore. Reagents used in this study were reagent grade or electrophoresis grade and were not repurified. Sequencing-grade reagents, glass fiber discs, Polybrene, sperm whale myoglobin, and bovine β-lactoglobulin were purchased from Applied Biosystems. Villin was purified from chicken intestine epithelial cells and cleaved with trypsin as described previously (9).

SDS-Polyacrylamide Gel Electrophoresis—Samples (10–250 pmol) were loaded onto minigels containing a 7.5–20% gradient of polyacrylamide and electrophoresed according to Laemmli (10) at 20 watts constant power (20 min). The minigels (10 × 10 cm, 0.5 mm thick) were modified from the original description of Matsudaira and Burgess (11). After electrophoresis, the gels were soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) for 5 min to reduce the amount of Tris and glycine. During this time a PVDF membrane was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was assembled into a blotting apparatus (Mighty Small, Hoeffer) and electroeluted for 10–30 min at 0.5 A in transfer buffer (12, 13). The PVDF membrane was washed in deionized H_2O for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 5–10 min at room temperature. The membrane was finally rinsed in deionized H_2O for 5–10 min, air dried, and stored at −20 °C.

Sequence Analysis—PVDF membranes were not precycled. Different amounts of protein (10–250 pmol) were spotted either on precycled Polybrene-coated trifluoroacetic acid-activated glass fiber filters or on small rectangles (2 × 4 mm) of PVDF membrane that were initially wetted with 100% acetonitrile. The membranes or filters were then air dried. Glass fiber filters were placed in the sequencing cartridge according to the manufacturer's instructions. Protein electroblotted onto PVDF membranes was stained with Coomassie Blue and the band was cut out with a clean razor. The membrane was centered on the Teflon seal and placed in the cartridge block of the sequenator. Proteins were sequenced on an Applied Biosystems model 470 sequenator equipped with on-line PTH analysis using the regular program O3RPTH. The PTH-derivatives were separated by reverse-phase HPLC over a Brownlee C-18 column (220 × 2.1 mm). The initial yield for the coupling step was calculated from the amount of PTH-derivatives present in the first cycle (corrected for 100% injection) and by the amount of protein spotted or electroblotted onto the membrane. The repetitive yield from myoglobin was determined from the peak heights of valine, leucine, and glutamic acid at positions 1, 2, 4, 11, 13, and 18. The repetitive yield from β-lactoglobulin was

* This work was supported by National Institutes of Health Grant AM35306, the Whitaker Foundation, and the PEW Scholars Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.

EXHIBIT L

calculated for leucine, isoleucine, and valine at residues 1, 2, 3, 10, 12, and 15. The repetitive yields for other proteins and peptides were calculated when possible from the recoveries of valine, alanine, or leucine residues.

RESULTS AND DISCUSSION

Proteins electroblotted onto PVDF membranes can be stained with Coomassie Blue, Amido Black, India Ink, or silver (8). As shown in Fig. 1, a 25-pmol digest of villin (2.4 μg) is easily detected by Coomassie Blue. The two major polypeptides, M_r 44,000 and 51,000, represent the NH_2- and COOH-terminal fragments of villin (9). Densitometry of Coomassie Blue-stained gels show both fragments are present in 5-pmol amounts. These fragments were cut from the PVDF membrane and subjected to 11 cycles of sequence analysis in a gas-phase sequenator. From the recovery of the PTH-derivatives in the first cycle, initial yields for coupling were 25–68%. Since complete transfer of the polypeptides to PVDF membranes was assumed, these values represent minimum estimate of the initial yields. The repetitive yields were 89–94%. The sequences agreed with those previously reported for the fragments.

Because preliminary experiments demonstrated that polypeptides electroblotted onto PVDF membranes can be readily sequenced, quantitative measurements of efficiency from these supports were made. Myoglobin and β-lactoglobulin were electroblotted onto PVDF membranes, and the recovery of PTH-derivatives was quantified. Fig. 2 shows the chromatograms of the PTH-derivatives released from 20 pmol of β-lactoglobulin for the first 15 cycles after electroblotting to PVDF membranes. As reported before (5, 6), Coomassie Blue does not interfere with the sequencing reactions nor produce artifact peaks on the HPLC traces. In the first 2 cycles, a variable amount of PTH-Gly was detected, but this amount is usually <5 pmol and reaches base-line levels after 2 cycles. Very little PTH-Gly is detected if the membranes are washed for 5–10 min in H_2O after transfer. The chromatograms display no DPU peak coeluting with PTH-Trp and the background levels of other PTH-derivatives remain low. Except for PTH-Trp, the recovery of PTH-derivatives was comparable to the amount obtained when Polybrene-coated glass fiber filters were used. Control experiments (not shown) indicate that PVDF membranes do not bind PTH-derivatives. When 100 pmol of PTH standards were applied directly to the membrane and the membrane washed, only 1–2 pmol of PTH-derivatives were detected. Very little PTH-Trp could be recovered from protein electroblotted onto the membranes. In contrast, PTH-Trp was detected in samples spotted onto the membrane. These facts indicate that tryptophan is destroyed either during electrophoresis or electroblotting.

The amount of material that yielded sequence was calculated from the initial yield for coupling, and the efficiency of the sequencing reaction was measured by the repetitive yield. Both values provide quantitative standards for sequence analysis from glass fiber discs and PVDF membranes. Table I shows that the average initial yield for proteins spotted onto PVDF membranes is 97% and for proteins electroblotted onto PVDF membranes is 76%. The latter value is more variable because of difficulties in accurately estimating the amount of protein present on the membrane. More important, the initial yields remain high even when the smallest amount of protein

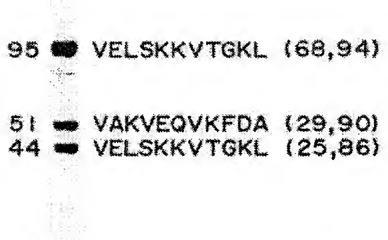

FIG. 1. **A tryptic digest of villin (2.4 μg of total protein) electroblotted to PVDF membranes and stained with Coomassie Blue.** Densitometry of digest shows that the intact villin band contains 12.5 pmol of protein, while the M_r 44,000 and 51,000 bands each contain 5 pmol of material. The bands were cut out and placed in the gas-phase sequenator. Their NH_2-terminal sequences are displayed in *1-letter code*. The initial yield for coupling and the repetitive yield are shown in *parentheses*. The repetitive yields were calculated from the recoveries of PTH-Val, PTH-Ala, and PTH-Leu.

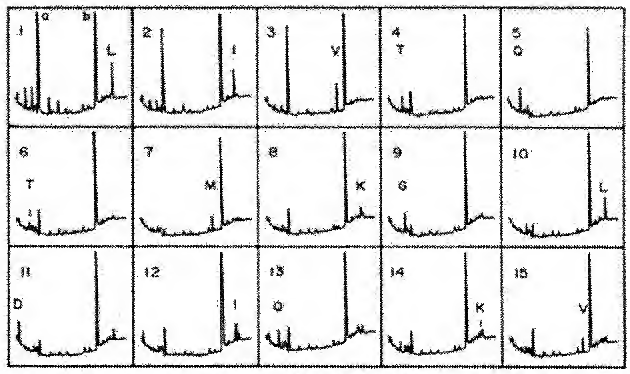

FIG. 2. **Chromatograms of PTH-derivatives, in *single letter abbreviation*, from 20 pmol of β-lactoglobulin electroblotted onto PVDF membranes and stained with Coomassie Blue.** The band was excised from the membrane and sequenced directly. The *abscissa* shows OD_{270}, the ordinate elution time. The peak height of leucine in cycle 1 corresponds to 6.7 pmol of injected material. Only 40% of the total PTH-derivatives is analyzed at every cycle. *Peak a* is N'N-dimethyl-N'-phenylthiourea; *peak b* is N'N-diphenylthiourea.

TABLE I

Comparison of the initial yield for coupling and repetitive yields for β-lactoglobulin and myoglobin from glass fiber and PVDF membranes

Initial and repetitive yields are calculated as described under "Experimental Procedures." The amount of protein listed in column 1 represents either the amount spotted onto the support or the calculated amount on the membrane after blotting (corrected for incomplete transfer).

	Support	Coupling yields	Repetitive yields
pmol		%	%
β-Lactoglobulin			
100	Polybrene-coated glass fiber	35	94.5
100	Spotted onto PVDF, unstained	108	90.7
200	Blotted onto PVDF, stained	75	93.0
50	Blotted onto PVDF, stained	100	93.3
20	Blotted onto PVDF, stained	84	92.8
Average		89	92.8
Myoglobin			
100	Polybrene-coated glass fiber	63	90.5
250	Spotted onto PVDF, unstained	88	87.8
100	Spotted onto PVDF, unstained	106	88.0
10	Spotted onto PVDF, unstained	86	88.0
66	Blotted onto PVDF, unstained	64	87.5
66	Blotted onto PVDF, stained	30	88.8
7	Blotted onto PVDF, stained	100	88.2
Average		79	88.1

TABLE II

Proteins and peptides sequenced from PVDF membranes

Proteins and peptides submitted to the Whitehead Protein Sequencing Facility were blotted or spotted on PVDF membranes. The sequenceable amount of material was determined from the recovery or the PTH-derivatives from the first cycle. The repetitive yield was calculated from the recovery of PTH-leucine, valine, or alanine. In some cases the repetitive yield was not determined (ND).

	Sequenceable amount		
	No. of cycles	pmol	Repetitive yield
			%
Proteins blotted to PVDF			
Villin (95 kDa)	11	8	87
Intestine 72-kDa protein	39	9	92
Intestine 105-kDa protein	6	3	96
Anion channel (105 kDa)	9	3	96
Anion channel 68-kDa fragment	10	15	ND
Anion channel 10-kDa fragment	16	17	94
Villin peptides blotted onto PVDF membranes			
44T (44 kDa)	12	2	92
44T-28C (28 kDa)	5	16	ND
51T (51 kDa)	11	2	90
51T-40C (40 kDa)	5	3	ND
51T-29C (29 kDa)	12	30	84
51T-28T (28 kDa)	5	8	ND
51T-16C (16 kDa)	12	16	ND
51T-8C (8 kDa)	5	16	ND
Proteins spotted onto PVDF membranes			
mutS (95 kDa)	5	50	ND
Thioredoxin (12 kDa)	10	9	ND
Homoserine kinase peptide (32 kDa)	10	30	ND
Methanogen hydrogenase (subunit a)	10	1000	98
Methanogen hydrogenase (subunit b)	18	300	ND
Methanogen hydrogenase (subunit c)	10	15	ND
Villin peptides spotted onto PVDF membranes[a]			
13V15 CNBr	8	20	ND
13V18 CNBr	3	25	ND
13V19 CNBr	3	25	ND
13V8 CNBr	7	60	ND
13V10 CNBr	6	63	ND
Synthetic peptides spotted onto PVDF membranes[b]			
CDTAGQEDYDRLR	5	ND	ND
RPDFSLEPPYTGPCK	12	ND	ND
PREDVDY	7	ND	ND

[a] Peptides from reverse-phase HPLC were generated by cyanogen bromide cleavage of M_r 13,000 peptide from villin.

[b] The sequences of the number of cycles during which the recovery of PTH-derivatives is at least 20% of the amount recovered in the first cycle are shown. In all cases, peptides which could not be completely sequenced were reanalyzed after absorption onto Polybrene-coated glass fiber discs or Polybrene-coated PVDF membranes.

is sequenced. The values compare extremely favorably with the 15–25% initial yields (6–7) measured for proteins electroblotted onto glass fiber filters. Since the binding capacity of PVDF membranes (170 $\mu g/cm^2$) (8) is greater than that of Polybrene-coated glass fiber sheets (15–28 $\mu g/cm^2$) (5) or derivatized glass fiber sheets (7–25 $\mu g/cm^2$) (6, 7), the high initial yields measured here were expected.

The high initial yields also suggest that NH_2-terminal modification during electrophoresis was not a major problem as no precautions were taken to remove contaminating chemicals. The gels were prepared in batch and stored for several days (1–7) before use, and samples were solubilized before electrophoresis by boiling in SDS sample buffer. Scavengers like thioglycolate were not included in the electrophoresis.

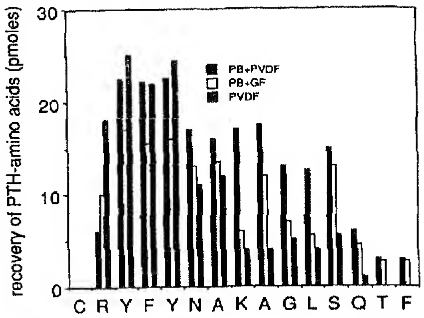

FIG. 3. **The recovery of PTH-derivatives is plotted from the analysis of peptide 8c which was spotted onto a PVDF membrane (*stippled bar*), PVDF membrane coated with Polybrene (*solid bar*), and glass fiber discs coated with Polybrene (*clear bar*).** Cysteine was not modified, and thus its recovery was not measureable. The amount of PTH-Ser was calculated from the recoveries of PTH-Ser and PTH-dehydro alanine.

The repetitive yields from proteins absorbed to glass fiber filters and PVDF membranes were similar, although the repetitive yield from PVDF membranes was 1–2% lower. The slightly reduced repetitive yield could result from a combination of incomplete cleavage, incomplete coupling, or peptide washout. The first two possibilities might be explained if the membrane interferes with the sequencing chemistry. However, the latter possibility is also likely since the hydrophobic binding of proteins and peptides to the membranes will be affected by the duration of the organic solvent washes in the Edman cycle. Because PVDF membranes have a 0.45-μm pore size and are thinner than a glass fiber disc, it is possible that the solvent wash steps in the sequencing programs are much longer than necessary for sequencing proteins absorbed to PVDF membranes. If true, then decreasing the duration of the solvent washes should improve the repetitive yields and shorten the cycle times.

The repetitive yields from myoglobin (90.5%) and β-lactoglobulin (94.5%) in the control experiments are lower than normal (usually 94 and 96%, respectively) and indicate that the sequenator was not operating at maximum efficiency. In addition to the test proteins reported here, these membranes have been used successfully to sequence 10- to 1000-pmol amounts of various proteins and peptides submitted to the Whitehead Facility. Table II shows that NH_2-terminal sequences can be obtained from other proteins (cytoplasmic and membrane) and polypeptides with a range of molecular weights. The repetitive yields ranged between 84–98%. In most cases repetitive yields were not calculated because the sequence lacked a repeated residue or the repetitive yield would be inaccurate due to poor recovery of some PTH-derivatives. The initial yields could not be measured since the band that was sequenced was from proteolytic digests composed of a mixture of peptides.

Short synthetic peptides were sequenced to examine whether peptide washout affects the recovery of PTH-derivatives. The results showed that complete analysis of short peptides can be problematic and depends on the hydrophobicity of the peptide. The recovery of PTH-derivatives from synthetic peptide 8c decreased by 80% after the Tyr-Phe-Tyr portion of the sequence when compared with the recovery from the same peptide spotted onto Polybrene-coated glass fiber discs or Polybrene-coated PVDF membranes (Fig. 3).

Other short peptides could be completely sequenced but the recovery of PTH-derivatives was variable and in poor yields. A common feature of the analysis of peptides was a large lag or carry over of each residue into the subsequent cycle. More reliable results were obtained by spotting the peptides on Polybrene-coated PVDF membranes or Polybrene-coated glass fiber discs. As a general rule, peptides that are too short to fractionate by polyacrylamide gel electrophoresis should be sequenced from Polybrene-coated supports. Proteins can be blotted in the presence of SDS and sequenced. Removal of SDS before analysis is advisable to prevent blockage of the delivery lines in the sequenator. The level of SDS is reduced by rinsing the membrane with H_2O.

This paper demonstrates that proteins can be efficiently sequenced after electroblotting and staining on PVDF membranes. There are several practical consequences that result from sequencing on these membranes. First, sample handling is simplified because the proteins can be stained after electroblotting. This eliminates the need to radiolabel or chemically modify the protein prior to electrophoresis. Second, the 3-h precycling step employed for conditioning Polybrene-coated glass fiber filters is eliminated, and thus the deadtime of the sequencing machine is reduced. Third, since Coomassie Blue can detect nanogram amounts of material and the repetitive yields and initial yield for coupling are not dependent on protein quantity, it should be possible to sequence femtomole amounts of proteins efficiently. However, improved sensitivity in detecting the cleaved residues will be needed to sequence these amounts of protein.

Acknowledgments—I would like to thank M. Wallek and S. Blackmon for their technical assistance and Dr. P. Kim for stimulating discussions.

REFERENCES

1. Elizinga, M. (ed). (1982) *Methods in Protein Sequence* Humana Press, Clifton, NJ
2. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997
3. Tarr, G. E., Beecher, J. F., Bell, M. & McKean, D. J. (1978) *Anal. Biochem.* **84**, 622–627
4. Laursen, R. A. (1971) *Eur. J. Biochem.* **20**, 89–102
5. Vandekerckhove, J., Bauw, G., Puype, M., Van Damme, J. & Van Montagu, M. (1985) *Eur. J. Biochem.* **152**, 9–19
6. Abersold, R. H., Teplow, D. B., Hood, L. E. & Kent, S. B. H. (1986) *J. Biol. Chem.* **261**, 4229–4238
7. Yuen, S., Hunkapiller, M. W., Wilson, K. J. & Yuan, P. M. (1986) *Applied Biosystems User Bulletin #24*, Foster City, CA
8. Pluskal, M. F., Przekop, M. B., Kavonian, M. R., Vecoli, C. & Hicks, D. A. (1986) *BioTechniques* **4**, 272–282
9. Matsudaira, P., Jakes, R. & Walker, J. E. (1985) *Nature* **315**, 248–250
10. Laemmli, U. K. (1970) *Nature* **227**, 680–685
11. Matsudaira, P. T. & Burgess, D. R. (1978) *Anal. Biochem.* **87**, 386–396
12. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
13. Burnette, W. N. (1981) *Anal. Biochem.* **122**, 195–203